Järvinen, M. (1979) *FEBS Lett. 108*, 461–464.
Kaplan, A. P. (1978) *Prog. Hemostasis Thromb. 4*, 127–175.
Kato, H., Nagasawa, S., & Iwanaga, S. (1981) *Methods Enzymol. 80*, 172–198.
Köhler, G., & Milstein, C. (1975) *Nature (London) 256*, 495–497.
Lenney, J. F., Liao, J. R., Sugg, S. L., Gopalakrishnan, V., Wong, H. C. H., Ouye, K. H., & Chan, P. W. H. (1982) *Biochem. Biophys. Res. Commun. 108*, 1581–1587.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Mandel, R., Colman, R. W., & Kaplan, A. P. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4179–4183.
Meier, H. L., Pierce, J. V., Colman, R. W., & Kaplan, A. P. (1977) *J. Clin Invest. 60*, 18–20.
Müller-Esterl, W., & Fritz, H. (1984) *Adv. Exp. Med. Biol. 167*, 41–62.
Müller-Esterl, W., Fritz, H., Machleidt, W., Ritonja, A., Brzin, J., Kotnik, M., Turk, V., Kellermann, J., & Lottspreich, F. (1985) *FEBS Lett. 182*, 310–314.
Ohkubo, I., Kurachi, K., Takasawa, T., Shiokawa, H., & Sasaki, M. (1984) *Biochemistry 23*, 5691–5697.
Ohkubo, I., Namikawa, C., Higashiyama, S., Sasaki, M., Minowa, O., Mizuno, Y., & Shiokawa, H. (1987) *Int. J. Biochem.* (in press).
Picard, C., Ronco, P., Moullier, P., Yao, J., Baudouin, B., Legendre, M. G., & Verroust, P. (1986) *Immunology 57*, 19–24.
Ryley, H. C. (1979) *Biochem. Biophys. Res. Commun. 89*, 871–878.
Salvesen, G., Parkes, C., Abrahamson, M., Grubb, A., & Barrett, A. J. (1986) *Biochem. J. 234*, 429–434.
Sasaki, M., Minakata, K., Yamamoto, H., Niwa, M., Kato, T., & Ito, N. (1977) *Biochem. Biophys. Res. Commun. 76*, 917–924.
Sasaki, M., Taniguchi, T., & Minakata, K. (1981) *J. Biochem. (Tokyo) 89*, 169–177.
Scott, C. F., Silver, L. D., Schapira, M., & Colman, R. W. (1984) *J. Clin. Invest. 73*, 954–962.
Subba Rao, P. V., McCartney-Francis, N. L., & Metcalfe, D. D. (1983) *J. Immunol. Methods 57*, 71–85.
Sueyoshi, T., Enjyoji, K., Shimada, T., Kato, H., Iwanaga, S., Bando, Y., Kominami, E., & Katunuma, N. (1985) *FEBS Lett. 182*, 193–195.
Sugo, T., Ikari, N., Kato, H., Iwanaga, S., & Fujii, S. (1980) *Biochemistry 19*, 3215–3220.
Tait, J. F., & Fujikawa, K. (1986) *J. Biol. Chem. 261*, 15396–15401.
Takagaki, Y., Kitamura, N., & Nakanishi, S. (1985) *J. Biol. Chem. 260*, 8601–8609.
Thompson, R. E., Mandle, R., & Kaplan, A. P. (1977) *J. Clin. Invest. 60*, 1376–1380.
Wakamatsu, N., Kominami, E., & Katunuma, N. (1982) *J. Biol. Chem. 257*, 14653–14656.

Spinach Chloroplast Fructose-1,6-bisphosphatase: Identification of the Subtilisin-Sensitive Region and of Conserved Histidines†

Frank Marcus,* Peter B. Harrsch,‡ Lorraine Moberly, Ida Edelstein, and Steven P. Latshaw
Department of Biological Chemistry and Structure, University of Health Sciences, The Chicago Medical School, North Chicago, Illinois 60064

Received April 24, 1987; Revised Manuscript Received June 29, 1987

ABSTRACT: Chloroplast fructose-1,6-bisphosphatase (FbPase) is an essential enzyme in the photosynthetic pathway of carbon dioxide fixation into sugars. The properties of the chloroplast enzyme are clearly distinct from those of cytosolic gluconeogenic FbPases. Light-dependent activation via a ferredoxin/thioredoxin system and insensitivity to inhibition by AMP are unique characteristics of the chloroplast enzyme. However, preliminary amino acid sequence data (78 residues) have demonstrated that a significant degree of amino acid sequence similarity exists between spinach chloroplast and mammalian gluconeogenic fructose-1,6-bisphosphatase [Harrsch, P. B., Kim, Y., Fox, J. L., & Marcus, F. (1985) *Biochem. Biophys. Res. Commun. 133*, 520–526]. In the present study, we have identified two structural features of spinach chloroplast FbPase that appear to be common to all FbPases. These include (a) the presence of a protease-sensitive area located in a region equivalent to residues 51–71 of mammalian FbPases and (b) the recognition of two conserved histidine residues, equivalent to histidines-253 and -311 of the mammalian enzymes. In addition, we have obtained sequence information accounting for more than three-fourths of the primary structure of spinach chloroplast FbPase. The high degree of homology observed between the chloroplast enzyme and gluconeogenic FbPases suggests a common evolutionary origin for all fructose-1,6-bisphosphatases in spite of their different functions and modes of regulation.

Fructose-1,6-bisphosphatase (FbPase)[1] catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate and inorganic phosphate. Because of its key role in gluconeogenesis, the enzyme has been extensively studied. Most of our knowledge on the subject comes from studies of the

† This work was supported by grants from the U.S. Department of Agriculture (83-CRCR-1-1299) and the National Institutes of Health (DK 21667 and DK 26564).

* Address correspondence to this author.
‡ Present address: Department of Medicinal Chemistry, Smith Kline and French Laboratories, Swedeland, PA 19479.

[1] Abbreviations: FbPase, fructose-1,6-bisphosphatase; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography; TFA, trifluoroacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PTH, phenylthiohydantoin; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; NADP, nicotinamide adenine dinucleotide phosphate; PTH, phenylthiohydantoin.

0006-2960/87/0426-7029$01.50/0

enzyme isolated from gluconeogenic tissues of animals [for reviews, see Benkovic and DeMaine (1981) and Tejwani (1982)]. The enzyme isolated from different sources is a tetramer composed of identical subunits of molecular weights ranging from 36 500 to 40 000. The complete amino acid sequence of FbPase from pig kidney cortex (Marcus et al., 1982) and sheep liver (Fisher et al., 1983) has been determined, and the comparison of these two structures reveals 90% sequence identity. Crystallographic studies of pig kidney FbPase have also been initiated (Seaton et al., 1984). More recently, additional structural information on the enzyme has been obtained from studies of the FbPase present in the yeast *Saccharomyces cerevisiae* (Rittenhouse et al., 1986; Rogers et al., 1987). Thus, structural information required to define FbPase structure/function relationships is beginning to emerge. In contrast, our knowledge of the enzyme present in plant tissues is much more limited. As with other enzymes involved in carbohydrate metabolism, plants contain a cytoplasmic/chloroplast isozyme pair (Weeden, 1981). The plant cytoplasmic FbPase participates in one of the sequence of reactions used by plants for sucrose synthesis from triose phosphates, and the enzyme has characteristics that are typical of gluconeogenic FbPases (Zimmermann et al., 1978). Chloroplast FbPase is essential in the photosynthetic pathway of carbon dioxide fixation into sugars [for reviews, see Buchanan (1980) and Halliwell (1981)], and the properties of the chloroplast enzyme are clearly distinct from cytosolic gluconeogenic FbPases. Insensitivity to AMP inhibition (Preiss et al., 1967; Buchanan et al., 1971) and light-dependent activation (Buchanan et al., 1979) are unique characteristics of the chloroplast enzyme.

Despite all differences, we first observed some similarities between chloroplast and gluconeogenic FbPases in their response to limited proteolysis by subtilisin (Marcus et al., 1980), and these observations led us to preliminary amino acid sequence studies which demonstrated some sequence similarity between spinach chloroplast and mammalian gluconeogenic FbPase (Harrsch et al., 1985). In the present work, we describe additional studies on the amino acid sequence of the major peptides obtained after tryptic digestion of control and subtilisin-treated spinach chloroplast FbPase. These studies have unveiled common structural features of the subtilisin-sensitive region and the recognition of two conserved histidine residues in all fructose-1,6-bisphosphatases so far known.

Experimental Procedures

Materials. The following materials were purchased from the sources indicated: DEAE-cellulose (DE-52) from Pierce; subtilisin Carlsberg, P 5380, from Sigma; hydroxylapatite, Bio-Gel HTP, from Bio-Rad. All peptides used as controls of the Pauly's test were from Sigma. These were Gly-His-Arg-Pro (G 8636), Tyr-Gly-Gly-Phe-Leu-NH_2 (E 3756), Asp-Arg-Val-Tyr-Ile-His-Pro-Phe (A 9525), and Ala-Ala-Ala (A 9627). Other reagents were purchased from common commercial sources and were of the highest purity available.

Purification of Spinach Chloroplast Fructose-1,6-bisphosphatase. The method described in detail below is a modification of the procedure described by Nishizawa et al. (1982). Unless otherwise noted, all of the operations were performed at 0–5 °C.

Step 1: Extract. Fresh market spinach (*Spinacea oleracea L.*) leaves (2 kg) were washed, drained of excess water, destemmed, placed in plastic bags, and frozen overnight at −15 °C. The frozen leaves were crumbled by hand and homogenized for 3 min with 2000 mL of 0.1 M potassium phosphate (pH 7.5) containing 2 mM EDTA in a large-capacity (3.8 L) three-speed blender set at the high-speed control (22 000 rpm). The homogenate was filtered through four layers of cheesecloth, and the filtrate was centrifuged for 25 min at 7000*g*.

Step 2: Ammonium Sulfate Fractionation. The pH of the above supernatant was adjusted to pH 7.5 with 0.1 N NaOH, and solid ammonium sulfate (31.3 g per 100 mL of solution) was slowly added. The suspension was stirred for 30 min and the precipitate removed by centrifuging for 30 min at 7000*g*. Solid ammonium sulfate (17.6 g per 100 mL of solution) was then added to the supernatant. After 30 min of equilibration, the precipitate was collected by centrifugation, dissolved in a minimum volume (not to exceed 50 mL) of 50 mM sodium acetate (pH 5.75) containing 0.25 mM EDTA, and dialyzed 2 times (for at least 3 h each) against 2 L of the above pH 5.75 buffer. The precipitate that formed during dialysis was removed by centrifuging.

Step 3: Sephadex G-100 Chromatography. The clarified fraction was applied to a Sephadex G-100 column (5 × 90 cm) equilibrated in and eluted with 50 mM sodium acetate (pH 5.75) contg. 0.25 mM EDTA. Fractions of 15 mL were collected and measured for protein (absorbancy at 280 nm) and FbPase activity. Fractions having more than 2 units/mL were pooled and diluted with an equal volume of 0.1 M HEPES–NaOH (pH 7.5) containing 0.25 mM EDTA. The pH was adjusted to 7.5 with 0.1 M NaOH, and the resulting solution was clarified by centrifugation.

Step 4: DEAE-cellulose Chromatography. The above fraction was applied to a 2.5 × 18 cm DEAE-cellulose (Whatman DE-52) column equilibrated 50 mM HEPES–NaOH buffer (pH 7.5) containing 0.25 mM EDTA and 0.1 M NaCl ("buffer A"). After application of the sample, the column was washed with buffer A until the absorbance of the effluent at 280 nm decreased to less than 0.02. Elution was then performed with a 500-mL linear gradient from 0.1 to 0.7 M NaCl in buffer A. Fractions of 10 mL were collected and measured for protein and FbPase activity. FbPase eluted beginning at about 0.33 M NaCl. Fractions having more than 5 units of FbPase per milliliter were pooled and dialyzed 2 times (for at least 2 h each) against 2 L of 50 mM sodium acetate, pH 5.75.

Step 5: Hydroxylapatite Chromatography. The above FbPase sample was applied to a hydroxylapatite column (2.5 × 8 cm) equilibrated in 50 mM sodium acetate, pH 5.75. Batchwise elutions were carried out sequentially with 100 mL each of 100, 200, 300, and 400 mM potassium phosphate (pH 5.7) in 50 mM sodium acetate, pH 5.75, and fractions of 10 mL were collected. The bulk of FbPase activity eluted with 200 and 300 mM potassium phosphate. Fractions having a specific activity greater than 60 units/mg were stored frozen in individual tubes at −15 °C until needed.

Enzyme Assay. FbPase activity was determined spectrophotometrically at 30 °C by following the rate of formation of NADPH at 340 nm in the presence of excess glucose-6-phosphate isomerase and glucose-6-phosphate dehydrogenase. Unless otherwise stated, the reaction mixture contained 40 mM Tris-HCl (pH 8.5), 0.6 mM fructose 1,6-bisphosphate, 10 mM $MgSO_4$, 0.1 mM EDTA, 0.3 mM NADP, and 1.2 units each of the auxiliary enzymes. The reaction was initiated by the addition of FbPase. Enzyme dilutions were made in 50 mM sodium acetate (pH 5.7) containing 0.25 mM EDTA. One unit of enzyme activity is defined as that amount of enzyme which catalyzes the formation of 1 μmol of fructose 6-phosphate/min. Specific activity is expressed as units per milligram of protein. FbPase concentration was determined by absorbance at 280 nm by using a $E^{1\%}_{1cm}$ value of 7.7 (Buchanan et

al., 1971), except that the protein concentrations during the initial steps of the enzyme purification were determined by a modified Biuret procedure (Mokrash & McGilvery, 1956).

Electrophoretic Techniques. SDS gel electrophoresis[2] was performed as previously described (Hosey & Marcus, 1981). In some of the runs, the protein was not reduced with mercaptoethanol before electrophoresis to visualize disulfide bonding (Allore & Barber, 1984). Molecular weight standards were from Bio-Rad, except for the addition of purified yeast (*S. cerevisiae*) FbPase (Rittenhouse et al., 1986).

Subtilisin Treatment. Chloroplast FbPase (274 μg) purified as described above, plus the additional FPLC step described under Results and Discussion, was treated for 60 min at 22 °C with subtilisin, at a w/w ratio of FbPase to subtilisin of 700:1 (Marcus et al., 1980). The reaction was stopped by the addition of 0.05 volume of 2 mM phenylmethanesulfonyl fluoride in absolute ethanol, and the solution was lyophilized.

S-Carboxymethylation and Tryptic Digestion. S-Carboxymethylation of chloroplast FbPase and of subtilisin-treated FbPase was performed as described by Crestfield et al. (1963) with minor modifications (Marcus et al., 1981), but standard dialysis tubing (molecular weight cutoff 12000–14000) was used for dialysis. Digestion of the S-carboxymethylated protein with trypsin (TRTPCK from Cooper/Worthington) was performed for 16 h at 22 °C in 50 mM *N*-ethylmorpholine–acetate buffer (pH 8.5) at a 50:1 ratio of FbPase to trypsin. The tryptic peptides were separated by reversed-phase HPLC as described in the legend to Figure 2.

Detection of Histidine-Containing Peptides. The volume of HPLC peptide peaks (containing about 2 nmol of peptide) was reduced to 10–20 μL by vacuum centrifugation. The sample was then applied on Whatman No. 1 filter paper. Four applications of 5 μL each were generally made, and the paper was air-dried between sample applications. The filter paper was then dipped in Pauly's A plus B solutions and allowed to dry in air for 5 min. The paper was then sprayed with solution C [for details about the reagents, see Allen (1981)]. Histidine-containing peptides gave a pale yellow color, while tyrosine-containing peptides gave a pink spot. Peptides containing both tyrosine and histidines gave orange colors. The sensitivity of this spot technique is about 2 nmol/cm^2. Appropriate control peptides (see Materials) containing histidine, tyrosine, both, or neither were always included as references.

Sequence and Amino Acid Analyses. Automated microsequencing of peptides (0.2–1.5 nmol) was performed in an Applied Biosystems 470A gas-phase protein sequencer using the standard sequencing program and the reagents provided by the manufacturer. The phenylthiohydantoin derivatives of amino acids liberated after each degradation cycle were identified and quantitated as such by HPLC using a modification of the procedure described by Hunkapiller (1985). The analyses were performed with a Waters Model 840 system equipped with an Altex Ultrasphere ODS 5-μm column (4.6 × 250 mm). Amino acid analyses were performed as described by Bidlingmeyer et al. (1985). Peptides (100–500 pmol) were hydrolyzed in vapor of 6 N HCl for 22 h at 110 °C. The liberated amino acids were reacted with phenyl isothiocyanate and the resulting derivatives analyzed by reversed-phase HPLC on a Waters 840 system equipped with a Waters Pico-Tag column.

Results and Discussion

Enzyme Purification. Spinach chloroplast FbPase was purified as described in detail under Experimental Procedures. The method that was developed is based on a previously published procedure (Nishizawa et al., 1982), but includes several modifications to improve both yield and reproducibility. Changes include some of the buffers used, the elimination of the pH 4.5 fractionation step, a significant modification of the DEAE-cellulose chromatographic step, and the elimination of the concentration procedures used by Nishizawa et al. (1982). The purification method described herein has been utilized many times in our laboratory to prepare relatively large batches of spinach chloroplast FbPase. The yield of about 13 mg of enzyme/kg of spinach leaves is significantly better than those of earlier procedures (Zimmermann et al., 1978; Nishizawa et al., 1982). The purity of the preparations, as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, is at least 95%. If homogeneous enzyme is needed, the remaining impurities can be removed by an additional step of fast protein liquid chromatography on a Pharmacia Mono Q HR 5/5 column equilibrated with buffer A. Chloroplast FbPase that had been dialyzed 2 times against 500 mL of buffer A is then applied to the column via a Pharmacia V-7 valve and a sample loop. After application of the sample, elution at a flow rate of 0.5 mL/min is performed with a linear gradient of NaCl (0.1–0.7 M in 40 min) in buffer A. Absorbance at 280 nm is continuously monitored, and chloroplast FbPase elutes as a sharp protein peak at about 0.36 M NaCl. Enzyme-containing fractions are pooled, dialyzed against buffer A, and then stored at −15 °C in 1-mL aliquots.

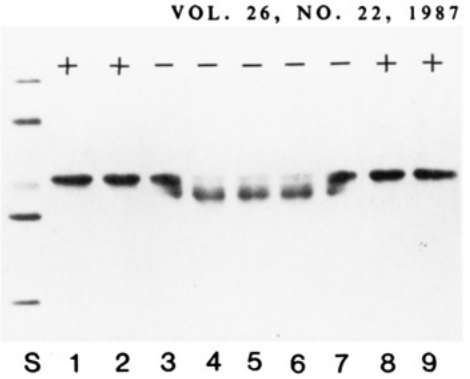

FIGURE 1: SDS gel electrophoresis of spinach chloroplast FbPase. The figure shows the Coomassie blue stained gel of purified enzyme (3.5 μg) electrophoresed with (+) or without (−) the addition of 5% mercaptoethanol to the samples. Lane S shows the following molecular weight markers: phosphorylase *b*, M_r 97000; bovine serum albumin, M_r 68000; ovalbumin, M_r 43000; yeast (*S. cerevisiae*) FbPase, M_r 38000; carbonic anhydrase, M_r 31000.

The purified enzyme was observed to migrate as a single band corresponding to a molecular weight of 44000 in SDS gel elecrophoresis (Figure 1, lanes 1, 2, 8, and 9). Previous estimates of the subunit molecular weight range from 35000 to 44000 (Buchanan et al., 1971; El Badry, 1974; Zimmermann et al., 1976; Marcus et al., 1980; Gontero et al., 1985). The sequencing studies currently in progress should provide a definite value of the molecular weight of the spinach chloroplast FbPase subunit. There are examples of significant value differences between estimates from SDS gel electrophoresis and sedimentation experiments and those determined from the known amino acid sequence (Takio et al., 1982). SDS gel electrophoresis was also used to test whether chloroplast FbPase contained inter- and/or intramolecular disulfide bonds (Allore & Barber, 1984). The results obtained with enzyme samples electrophoresed with or without the addition of mercaptoethanol showed that the mobility of chloroplast FbPase was higher in the absence of the reducing agent mercaptoethanol (Figure 1, lanes 4–6), indicating the presence

[2] Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate will herein be referred to as SDS gel electrophoresis.

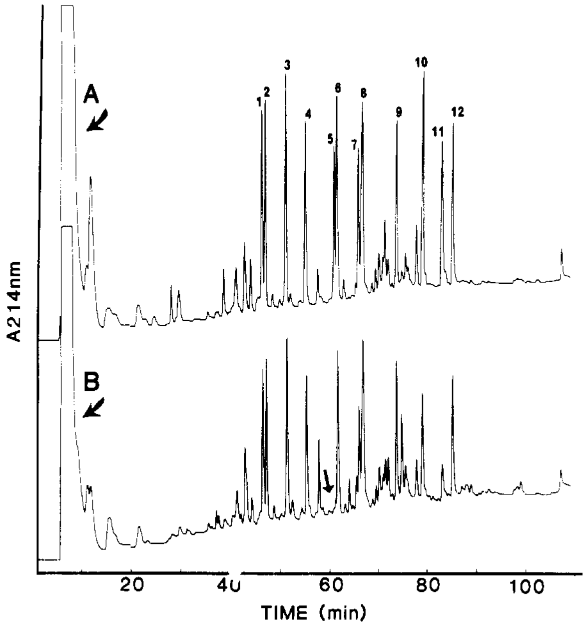

FIGURE 2: Reversed-phase HPLC of a tryptic digest of control (A) and subtilisin-treated (B) S- arboxymethylated spinach chloroplast FbPase. The sample was acidified to pH 4 with concentrated acetic acid, and 1–2 nmol was injected into a Bio-Rad RP-304 column (4.6 × 250 mm) equilibrated with 0.1% TFA. Peptides were eluted with an H_2O/acetonitrile gradient containing 0.1% TFA (0% to 45% acetonitrile over a period of 90 min) and a flow rate of 0.5 mL/min. The major peaks separated in chromatograph A were numbered on the basis of their order of elution. A straight arrow at about 61 min in chromatograph B is shown to indicate the disappearance of peptide 5 as the result of subtilisin treatment.

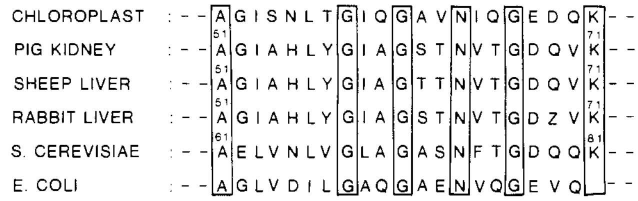

FIGURE 3: Sequence homology at the subtilisin-sensitive region of fructose-1,6-bisphosphatases. Amino acids are indicated by the single-letter code, and identical residues are shown in boxes. The sequence data of pig kidney, sheep liver, rabbit liver, *S. cerevisiae*, and *E. coli* FbPase are taken from Marcus et al. (1982), Fisher and Thompson (1983), MacGregor et al. (1982), Rogers et al. (1987), and Marcus et al. (1986), respectively. *Numbers* above residues indicate their location in the corresponding amino acid sequence. Ala-51 and Gly-58 are also present in rat liver and rabbit, chicken, and snake muscle FbPases, and Gly-61, Asx-64, Gly-67, and Lys-71 have been reported for rat liver and rabbit muscle FbPases (MacGregor et al., 1982).

of intrasubunit disulfide bonds. More than one band was observed in the absence of mercaptoethanol, which is probably due to the presence of more than one intrasubunit disulfide bond, in agreement with previous reports (Pradel et al., 1981). The pattern shown in lanes 3 and 7 (Figure 1) is typical of the intermediate zone with a downward transition due to mixtures of fully reduced and nonreduced states (Allore & Barber, 1984). There appears to be, however, no evidence for the existence of intersubunit disulfide bonds (Pradel et al., 1981; Gontero et al., 1985), which should have shown up as higher molecular weight bands on SDS gel electrophoresis of the enzyme samples without mercaptoethanol (Allore & Barber, 1984).

Identification of the Subtilisin-Sensitive Region. As described (Marcus et al., 1980), limited proteolysis of spinach chloroplast FbPase with subtilisin results in the formation of a nicked form of the enzyme that exhibits reduced enzyme activity measured at pH 8.3 as the consequence of a shift of the pH optimum to a more alkaline pH. Since a similar effect as the result of limited proteolysis appears to be a characteristic of a variety of mammalian FbPases (Horecker et al., 1975; Marcus, 1981; MacGregor et al., 1982) and in each case proteolysis occurs in a well-established proteinase-sensitive region, it appeared of importance to determine the location of the subtilisin-sensitive region of chloroplast FbPase. With this purpose, chloroplast FbPase was treated with subtilisin to produce cleavage in the sensitive area, and tryptic peptides generated from the S-carboxymethylated subtilisin-treated chloroplast FbPase were isolated by HPLC (Figure 2B). The peptide pattern was compared to that obtained in a control experiment in which the subtilisin treatment had been omitted (Figure 2A). The pattern comparison showed that subtilisin treatment resulted in the complete disappearance of a tryptic peptide (peptide 5) that eluted at about 61 min in the control experiment A. The amino acid sequence analysis of peptide 5 gave the sequence Ala-Gly-Ile-Ser-Asn-Leu-Thr-Gly-Ile-Gln-Gly-Ala-Val-Asn-Ile-Gln-Gly-Glu-Asp-Gln-Lys (Table I), which shows 48% identity with the sequence of residues 51–71 of pig kidney FbPase. This is precisely the location of the subtilisin-sensitive region, known to be a common feature of vertebrate FbPases (MacGregor et al., 1982). However, conservation of sequence in this area appears to occur in all FbPases (Figure 3) with residues equivalent to Ala-51, Gly-58, Gly-61, Asn-64, Gly-67, and Lys-71 of mammalian FbPases always present.

Sequence of Major Tryptic Peptides. As shown in Figure 2A, the HPLC separation of the tryptic peptides obtained from S-carboxymethylated spinach chloroplast FbPase yielded a pattern containing 12 major peaks. These peaks, designated by *arabic numerals* on the basis of their order of elution, were selected for sequence analysis. The amino acid sequence data obtained for all 12 peptides is given in Table I, and the data show that 11 of the 12 peptides had amino acid sequences that were easily aligned by using as a frame of reference the amino acid sequence for pig kidney FbPase (Marcus et al., 1982). The alignment did not require the introduction of deletions or insertions and revealed sequence homologies ranging from 29% to 75%. Some of the tryptic peptides, peptides 7, 10, and 12, had previously sequenced under former designations of T-16, T-21, and T-23, respectively (Harrsch et al., 1985).

Tryptic peptide 1 was the only peptide that was not aligned by sequence comparison with the structure of pig kidney FbPase. However, we have recently established that it corresponds to the sequence of residues 218–225 of pig kidney FbPase although its degree of homology is only 12.5%. The information concerning the location of this peptide was obtained from the nucleotide sequence determined from a recombinant cDNA clone containing this region of pea chloroplast FbPase (H. T. Smith and F. Marcus, unpublished experiments). The sequence information given in Table I, plus that contained in a previous report (Harrsch et al., 1985), raises the number of sequenced amino acids to 182. Preliminary data, including the sequence of some additional fragments (Marcus & Harrsch, 1987), have extended our knowledge of the sequence of spinach chloroplast FbPase to a total of 285 residues and reveal 46% homology between pig kidney FbPase and the spinach chloroplast enzyme. This high degree of homology strengthens our previous suggestion of a common evolutionary origin for a variety of gluconeogenic FbPases and the chloroplast FbPase (Harrsch et al., 1985; Marcus et al., 1986), enzymes catalyzing the same reaction but having different functions and modes of regulation.

Table I: Sequence Analysis of Peptide Fragments Generated by Trypsin Treatment of Spinach Chloroplast FbPase[a]

cycle no.	PTH-amino acids from peptide											
	1	2	3	4	5	6	7	8	9	10	11	12
1	Met (452)	Tyr (610)	Ile (1161)	Ile (336)	Ala (361)	Tyr (496)	Lys (846)	Thr (198)	Val (927)	Leu (444)	Ser (150)	Gly (214)
2	Trp (213)	Met (510)	Tyr (1243)	Leu (383)	Gly (209)	Ile (332)	Leu (821)	Leu (906)	Pro (797)	Leu (340)	Lys (230)	Val (183)
3	Asp (771)	Asp (771)	Ser (404)	Asp (249)	Ile (292)	Gly (437)	Asp (274)	Leu (854)	Leu (802)	Tyr (391)	Tyr (206)	Tyr (213)
4	Asp (791)	Asp (791)	Phe (848)	Ile (279)	Ser (46)	Ser (208)	Val (675)	Tyr (740)	Tyr (680)	Glu (313)	Glu (168)	Ala (211)
5	Lys (179)	Leu (371)	Asn (828)	Gln (250)	Asn (206)	Leu (287)	Val (724)	Gly (536)	Ile (622)	Cys (209)	Ile (108)	Phe (181)
6	Leu (243)	Lys (380)	Glu (621)	Pro (261)	Leu (429)	Val (248)	Ser (228)	Gly (475)	Gly (529)	Ala (238)	Glu (136)	Thr (109)
7	Lys (212)	Glu (375)	Gly (485)	Thr (177)	Thr (124)	Gly (283)	Asn (480)	Ile (776)	Ser (212)	Pro (179)	Thr (31)	Leu (139)
8	Lys (57)	Pro (375)	Asn (611)	Glu (192)	Gly (213)	Asp (171)	Glu (339)	Tyr (857)	Val (427)	Met (184)	Leu (71)	Asp (84)
9		Gly (255)	Tyr (556)	Ile (173)	Ile (265)	Phe (273)	Val (459)	Gly (444)	Glu (358)	Ser (60)	Thr (28)	Pro (132)
10		Glu (277)	Lys (347)	His (63)	Gln (207)	His (151)	Phe (469)	Tyr (767)	Glu (362)	Phe (91)	Gly (48)	Met (126)
11		Ser (92)		Gln (128)	Gly (169)	Arg (17)	Ser (132)	Pro (369)	Val (272)	Ile (102)	Trp (13)	Tyr (97)
12		Gln (136)		Arg (16)	Ala (209)		Ser (112)	Arg (116)	Glu (289)	Val (65)	Leu (24)	Gly (76)
13		Lys (137)			Val (218)		Cys (107)		Lys (277)	Glu (55)	Leu (31)	Glu (55)
14		Pro (194)			Asn (174)		Leu (221)		Leu (219)	Gln (60)	Lys (22)	Phe (66)
15		Tyr (156)			Ile (192)		Arg (12)		Glu (171)	Ala (46)		Val (51)
16		Ser (48)			Gln (281)					Gly (42)		Thr (19)
17		Ser (55)			Gly (199)					Gly (42)		Thr (19)
18		Arg (14)			Glu (217)					Lys (23)		Ser (9)
19					Asp (218)							Glu (22)
20					Gln (184)							Lys (17)
21					Lys (56)							
residue nos.[b]		226–243	208–217	302–313	51–71	244–254	72–86	255–266	314–328	277–294	6–19	180–199
% homology		33	70	75	48	64	52	67	33	61	29	50

[a] Amino acid sequences of peptides, numbered as indicated in Figure 2A, were determined as described under Experimental Procedures. Recoveries in picomoles are given in parentheses. [b] These numbers indicate the corresponding location in the amino acid sequence of pig kidney FbPase (Marcus et al. 1982).

Table II: Sequences around Histidine Residues in Fructose-1,6-bisphosphatases

source of FbPase	sequence	ref
	253[a]	
pig kidney	-Ala-Asp-Val-His-Arg-Thr-Leu-	*b*
sheep liver	-Ala-Asp-Val-His-Arg-Thr-Leu-	*c*
yeast[i]	-Ala-Asp-Val-His-Arg-Thr-Phe-	*d*
E. coli	-Ala-Asp-Phe-His-Arg	*d*
chloroplast	-Gly-Asp-Phe-His-Arg-Thr-Leu-	this work
	311	
pig kidney	-Thr-Asp-Ile-His-Gln-Arg-Ala-	*b*
sheep liver	-Thr-Asp-Ile-His-Gln-Lys-Val-	*c*
yeast	-Ser-His-Ile-His-Asp-Lys-Ser-	*e*
E. coli	-Glu-Thr-Leu-His-Gln-Arg	*d*
chloroplast	-Thr-Glu-Ile-His-Gln-Arg-Val	this work and *f*
	55	
pig kidney	-Gly-Ile-Ala-His-Leu-Tyr-Gly	*b*
sheep liver	-Gly-Ile-Ala-His-Leu-Tyr-Gly	*c*
yeast	-Glu-Leu-Val-Asn-Leu-Val-Gly	*e*
E. coli	-Gly-Leu-Val-Asp-Ile-Leu-Gly	*d*
chloroplast	-Gly-Ile-Ser-Asn-Leu-Thr-Gly	this work
	334	
pig kidney	-Tyr-Gln-Lys-His-Ala	*b*
sheep liver	- - -Lys-Lys-Tyr-Thr-Ala	*c*
rabbit liver	-Tyr-Lys-Lys-His-Ala-Val-Lys	*g*
rat liver	-Tyr-Asx-Lys-Asp-Lys-Ala-Lys-	*h*

[a] The numbers above the histidine residues correspond to their location in the amino acid sequence of pig kidney FbPase. [b] Marcus et al., 1982. [c] Fisher & Thompson, 1983. [d] Marcus et al., 1985. [e] Rogers et al., 1987. [f] Harrsch et al., 1985. [g] Xu et al., 1982. [h] Rittenhouse et al., 1983. [i] Yeast denotes *S. cerevisiae*.

The results shown in Table I also serve to confirm that the subtilisin-sensitive area of chloroplast FbPase is located closer to the NH_2 terminus than to the COOH terminus of the enzyme. Indeed, peptide 11 shows a sequence of residues corresponding to residues 6–19 of pig kidney FbPase, and it is clear that the amount of this peptide is greatly decreased in chromatograph B of Figure 2. A decrease in peptides derived from the low molecular weight fragment produced by subtilisin treatment of chloroplast FbPase (Marcus et al., 1980) is to be expected since dialysis after S-carboxymethylation (see Experimental Procedures) was performed with the standard tubing which has a molecular weight cutoff of 12000–14000.

Identification of Conserved Histidine Residues. The presence of histidine residues in peptide peak numbers 4 and 6 of Figure 2A was recognized prior to sequence analysis by a convenient and sensitive spot detection method described in detail under Experimental Procedures. The sequence analysis indeed confirmed that these were two histidine-containing peptides and at the same time revealed that these two peptides were among those having the highest degree of sequence homology with pig kidney FbPase. Peptide 4 exhibited 75% amino acid identity with the sequence of residues 302–313 of pig kidney FbPase, while peptide 6 showed 64% identity with the sequence of residues 244–254 of the pig kidney enzyme. Thus, two histidine residues of the spinach chloroplast enzyme were identified as being equivalent to His-253 and His-311 of pig kidney FbPase. An inspection of these sequence regions in several known FbPases suggests that these two histidine residues are conserved in all FbPases (Table II). By contrast, the two other histidine residues found in pig kidney FbPase, His-55 and His-334, are not always conserved. It is tempting to speculate that the conserved histidine residues of FbPase are related to their yet undetermined functional role. One likely possibility could releate to participation in Zn binding to FbPase (Pedrosa et al., 1977; Benkovic et al., 1978; Pontremoli et al., 1978). There are several well-known examples of other enzymes, i.e., thermolysin (Colman et al., 1972),

carboxypeptidase A (Vallee et al., 1983), *Escherichia coli* alkaline phosphatase (Sowadski et al., 1985), and alcohol dehydrogenase (Eklund et al., 1976), in which histidine residues are ligands to zinc. Although an essential role for histidines in FbPases has not been yet established, the modification of histidine residues with ethoxyformic anhydride results in the inactivation of rabbit liver (DeMaine & Benkovic, 1980), pig kidney, and spinach chloroplast (I. Edelstein and F. Marcus, unpublished experiments) FbPases. It is also of interest to note that recent work by Scheffler and Fromm (1986) has shown that two His C-2 protons of rabbit liver FbPase are well resolved in the aromatic ^{1}H NMR spectrum of the enzyme and that distinct spectral perturbations occur upon fructose 1,6-bisphosphate, fructose 2,6-bisphosphate, and AMP binding to the enzyme. It would appear important to establish whether these two perturbable histidines correspond to the two histidine residues conserved in all FbPases.

CONCLUSIONS

The results presented in this paper have lead to the identification of two features of spinach chloroplast FbPase that appear to be common to all FbPases. These are (a) the presence of a protease-sensitive area located in a region equivalent to residues 51–71 of mammalian FbPases and (b) the presence of two conserved histidine residues, equivalent to histidines-253 and -311 of the mammalian enzymes. In addition, we have completed sequence information accounting for more than three-fourths of the primary structure of spinach chloroplast FbPase. The high degree of homology observed between the chloroplast enzyme and gluconeogenic FbPases strengthens our view (Marcus et al., 1986) of a common evolutionary origin for all fructose-1,6-bisphosphatases in spite of their different functions and modes of regulation.

ACKNOWLEDGMENTS

We thank Dr. Judith Rittenhouse for helpful discussions.

Registry No. FbPase, 9001-52-9; L-His, 71-00-1.

REFERENCES

Allen, G. (1981) in *Sequencing of Proteins and Peptides*, p 158, Elsevier, Amsterdam.

Allore, R. J., & Barber, B. H. (1984) *Anal. Biochem. 137*, 523–527.

Benkovic, P. A., Caperelli, C. A., DeMaine, M. M., & Benkovic, S. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2185–2189.

Benkovic, S. J., & DeMaine, M. M. (1981) *Adv. Enzymol. Relat. Areas Mol. Biol. 53*, 45–82.

Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. L. (1985) *J. Chromatogr. 336*, 93–104.

Buchanan, B. B., Schurmann, P., & Kalberer, P. P. (1971) *J. Biol. Chem. 246*, 5952–5959.

Buchanan, B. B., Wolosiuk, R. A., & Schurmann, P. (1979) *Trends Biochem. Sci. (Pers. Ed.) 4*, 93–96.

Colman, P. M., Jansonius, J. N., & Mathews, B. W. (1972) *J. Mol. Biol. 70*, 701–724.

Crestfield, A. M., Moore, S., & Stein, W. H. (1963) *J. Biol. Chem. 238*, 622–627.

DeMaine, M. M., & Benkovic, S. J. (1980) *Arch. Biochem. Biophys. 205*, 308–314.

Eklund, H., Branden, C.-I., & Jornvall, H. (1976) *J. Mol. Biol. 102*, 61–73.

El-Badry, A. M. (1974) *Biochim. Biophys. Acta 333*, 366–377.

Fisher, W. K., & Thompson, E. O. P. (1983) *Aust. J. Biol. Sci. 36*, 235–250.

Gontero, B., Meunier, J. C., Sauve, P., & Ricard, J. (1985) *Plant Sci. Lett. 38*, 17–22.

Halliwell, B. (1981) in *Chloroplast Metabolism*, pp 66–88, Oxford University Press, New York.

Harrsch, P. B., Kim, Y., Fox, J. L., & Marcus, F. (1985) *Biochem. Biophys. Res. Commun. 133*, 520–526.

Horecker, B. L., Melloni, E., & Pontremoli, S. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 42*, 193–226.

Hosey, M. M., & Marcus, F. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 91–94.

Hunkapiller, M. W. (1985) *Protein Sequencer User Bulletin No. 14*, Applied Biosystems, Foster City, CA.

Marcus, F., & Harrsch, P. B. (1987) in *Progress in Photosynthesis Research* (Biggins, J., Ed.) Vol. 3, pp 471–474, Martinus Nijhoff, Dordrecht, The Netherlands.

Marcus, F., Edelstein, I., Nishizawa, A. N., & Buchanan, B. B. (1980) *Biochem. Biophys. Res. Commun. 97*, 1304–1310.

Marcus, F., Edelstein, I., Saidel, L. J., Keim, P. S., & Heinrikson, R. L. (1981) *Arch. Biochem. Biophys. 209*, 687–696.

Marcus, F., Edelstein, I., Reardon, I., & Heinrikson, R. L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7161–7165.

Marcus, F., Gontero, B., Harrsch, P. B., & Rittenhouse, J. (1986) *Biochem. Biophys. Res. Commun. 135*, 374–381.

McGregor, J. S., Hannappel, E., Xu, G.-J., Pontremoli, S., & Horecker, B. L. (1982) *Arch. Biochem. Biophys. 217*, 652–664.

Mokrasch, L. C., & McGilvery, R. W. (1956) *J. Biol. Chem. 221*, 909–917.

Nishizawa, A. N., Yee, B. C., & Buchanan, B. B. (1982) in *Methods in Chloroplast Molecular Biology* (Edelman, M., Hallick, R. B., & Chua, N.-H., Eds.) pp 707–713, Elsevier Biomedical, Amsterdam.

Pedrosa, F. O., Pontremoli, S., & Horecker, B. L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2742–2745.

Pontremoli, S., Melloni, E., Salamino, F., Sparatore, B., & Horecker, B. L. (1978) *Arch. Biochem. Biophys. 188*, 90–97.

Pradel, J., Soulie, J. M., Buc, J., Meunier, J. C., & Ricard, J. (1981) *Eur. J. Biochem. 113*, 507–511.

Preiss, J., Biggs, M. L., & Greenberg, E. (1967) *J. Biol. Chem. 242*, 2292–2294.

Rittenhouse, J., Chatterjee, T., Marcus, F., Reardon, I., & Heinrikson, R. L. (1983) *J. Biol. Chem. 258*, 7648–7652.

Rittenhouse, J., Harrsch, P. B., Kim, J. N., & Marcus, F. (1986) *J. Biol. Chem. 261*, 3939–3943.

Rogers, D. T., Hiller, E., Rittenhouse, J., Moberly, L., Edelstein, I., & Marcus, F. (1987) *Protein Eng. 1*, 257.

Scheffler, J. E., & Fromm, H. J. (1986) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 45*, 1868.

Seaton, B. A., Campbell, R. L., Petsko, G. A., Rose, D. R., Edelstein, I., & Marcus, F. (1984) *J. Biol. Chem. 259*, 8915–8916.

Sowadski, J. M., Handschumacher, M. D., Krishna Murthy, H. M., Foster, B. A., & Wycoff, H. W. (1985) *J. Mol. Biol. 186*, 417–433.

Takio, K., Smith, J. B., Krebs, E. G., Walsh, K. A., & Titani, K. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2544–2548.

Tejwani, G. A. (1982) *Adv. Enzymol. Relat. Areas Mol. Biol. 54*, 121–194.

Vallee, B. L., Galdes, A., Auld, D. S., & Riordan, J. F. (1983) in *Zinc Enzymes* (Spiro, T. G., Ed) pp 25–75, Wiley-Interscience, New York.

Weeden, N. F. (1981) J. Mol. Evol. 17, 133–139.
Xu, G.-J., Natalini, P., Suda, H., Tsolas, O., Dzugaj, A., Sun, S. C., Pontremoli, S., & Horecker, B. L. (1982) Arch. Biochem. Biophys. 214, 688–694.
Zimmermann, G., Kelly, G. J., & Latzko, E. (1976) Eur. J. Biochem. 70, 361–367.
Zimmermann, G., Kelly, G. J., & Latzko, E. (1978) J. Biol. Chem. 253, 5952–5956.

Bovine Cardiac Troponin T: Amino Acid Sequences of the Two Isoforms†

John Leszyk,‡ Ranjana Dumaswala,§ James D. Potter,‖ Nikolai B. Gusev,⊥ Alexandr D. Verin,⊥ Larry S. Tobacman,# and John H. Collins*,‡

Departments of Biology and Chemistry, Clarkson University, Potsdam, New York 13676, Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267, Department of Pharmacology, University of Miami School of Medicine, Miami, Florida 33101, Department of Biochemistry, Moscow State University, Moscow, USSR, and Department of Internal Medicine, University of Iowa, Iowa City, Iowa 52242

Received April 16, 1987; Revised Manuscript Received June 19, 1987

ABSTRACT: Troponin T (TnT) is the tropomyosin-binding subunit of troponin, the thin filament regulatory complex that confers calcium sensitivity to striated muscle contraction and actomyosin ATPase activity. Bovine cardiac muscle contains two isoforms (TnT-1 and TnT-2) of TnT that differ in sequence near their amino termini. Thin filaments containing TnT-2 require less calcium to activate the MgATPase rate of myosin than do thin filaments containing TnT-1. Using whole troponin T purified from adult bovine cardiac muscle, we have determined the complete amino acid sequence of the larger, more abundant isoform TnT-1. We confirmed that sequence differences between TnT-1 and TnT-2 are confined to the amino-terminal regions and found that TnT-1 makes up approximately 75% of the total troponin T isolated. Partial sequencing of the separated isoforms showed that the difference between them is due solely to residues 15–19 (Glu-Ala-Ala-Glu-Glu) of TnT-1 being absent from TnT-2. The deleted segment may correspond to the product of exon 4 of the chicken cardiac TnT gene [Cooper, T. A., & Ordahl, C. P. (1985) J. Biol. Chem. 260, 11140–11148]. Exon 5, which is developmentally regulated in the chicken, is not expressed in either TnT-1 or TnT-2. TnT-1 contains 284 amino acid residues and has a M_r of 33 808, while TnT-2 contains 279 amino acid residues and has a M_r of 33 279. Bovine cardiac TnT contains the only known thiol group in any isolated TnT (Cys-39 of TnT-1, Cys-34 of TnT-2). Comparison of bovine, rabbit, and chicken cardiac TnT sequences shows near identity of the amino-terminal 13 amino acid residues (exons 2 and 3 of the chicken cardiac gene), many differences in the following 60 residues (exons 4–8), and great similarity in the C-terminal 230 residues (exons 9–18).

The interaction of actin and myosin that occurs during muscle contraction is regulated by changes in intracellular Ca^{2+} concentration. In vertebrate striated muscles the dominant regulatory system involves binding of Ca^{2+} to troponin in the thin filaments. Troponin is a complex of three different protein subunits: TnC binds Ca^{2+}, TnI binds to actin and inhibits actin–myosin interaction, and TnT binds to tropomyosin. The amino acid sequences of all three rabbit fast skeletal muscle troponin subunits have been determined (Wilkinson & Grand, 1974; Collins et al., 1973, 1977; Pearlstone et al., 1976, 1977), and these proteins have served as the models for extensive structure–function studies carried out in several laboratories [see Leavis and Gergely (1984) for review]. Of particular interest for the present study is the manner in which TnT interacts with tropomyosin. The state of our knowledge in this area was recently summarized by White et al. (1987), who have carried out X-ray diffraction studies of the rabbit skeletal muscle troponin–tropomyosin complex. Their results are consistent with biochemical studies, which suggested a two-site model for the attachment of TnT to tropomyosin. TnT is very asymmetric and binds along the length of the carboxyl-terminal third of the tropomyosin molecule in an antiparallel fashion. The amino terminus (residues 1–70) of TnT binds to the carboxyl terminus of tropomyosin and also spans the head-to-tail junction between adjacent tropomyosin molecules in the thin filament, interacting with a small amino-terminal region of the adjacent tropomyosin molecule. This interaction of tropomyosin and the amino-terminal region of TnT forms an invariant, Ca^{2+}-insensitive linkage that is important in maintaining the cooperativity of thin filament proteins. Residues 71–158 of TnT are highly α-helical and extend toward the middle of the tropomyosin molecule, probably forming electrostatic interactions with tropomyosin. The carboxyl-terminal region of TnT binds near residues 150–180 of tropomyosin, ~20 nm away from the head-to-tail junction. In the whole troponin complex, the carboxyl-terminal region of TnT also binds TnC and TnI, and the linkage between TnT and tropomyosin becomes Ca^{2+} sensitive. Binding of Ca^{2+} to TnC apparently breaks this TnT–tropomyosin link and allows the tropomyosin

† This work was supported by NIH Grants AR35120 (J.H.C.) and HL22619-3A (J.D.P.).
* Address correspondence to this author at the Department of Biological Chemistry, School of Medicine, University of Maryland at Baltimore, Baltimore, MD 21201.
‡ Clarkson University.
§ University of Cincinnati College of Medicine.
‖ University of Miami School of Medicine.
⊥ Moscow State University.
University of Iowa.

0006-2960/87/0426-7035$01.50/0